BINDING OF PARATROPOMYOSIN TO CONNECTIN/TITIN DOMAINS LOCATED AT THE A-I JUNCTION OF CHICKEN SARCOMERES

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Introduction

A myofibrillar protein, paratropomyosin weakens the rigor linkages between actin and myosin, and contributes to meat tenderization. Paratropomyosin was found at the A-I junction of sarcomeres in living muscle and in muscle immediately postmortem, and translocated from its original position to thin filaments by an increase of calcium ion concentration to 0.1 mM during postmortem storage of muscles (Hattori and Takahashi, 1988). We have shown that in chicken breast muscle the binding of paratropomyosin to both b-connectin/titin 2 and the 43-kDa fragment, the proteolytic product of b-connectin/titin 2, in examining binding of paratropomyosin at the A-I junction, and determined the N-terminal sequence of the fragment (Fei, et al., 1999; Yamanoue et al., 2003). Also we have cloned DNA fragment encoding the 43-kDa fragment and produced in *E. coli* a recombinant I53 domain (rCK-I53) that constituted the fragment. An image of indirect immunofluorescence microscopy showed that the rCK-I53 domain was located at the A-I junction of chicken sarcomeres (Yamanoue et al., 2005).

In the present study, we produced all recombinant domains that constituted the 43-kDa fragment and examined binding of paratropomyosin to each domain on PVDF membrane in order to identify more precise binding site in intact connectin/titin molecule.

Materials and Methods

Expression of recombinant connectin/titin domains. For construction of expression vectors, DNA fragments encoding a single and two successive domains were amplified by PCR using a set of specific primers based on the nucleotide sequence of the chicken 43-kDa fragment and cloned into the *Nde* I - *Bam*H I site of the expression vector, pET- 22b (Novagen, USA). After the expression vectors were introduced into the *E. coli* BL21 (DE3) competent cell, production of the recombinant domains were induced by the addition of isopropyl-b-d-thiogalactopyranoside (IPTG). Transformed cells were harvested and sonicated. The cell lysates were centrifuged and the supernatants were successively applied to gel filtration and hydrophobic interaction chromatography.

Overlay assay. Purified recombinant domains or the cell lysates were applied to SDS-PAGE and then electrotransferred to PVDF membranes. The separated proteins were overlaid with paratropomyosin and then incubated with anti-paratropomyosin antiserum. Binding of paratropomyosin to the domains was detected by alkaline phosphatase (AP)-labeled anti-rabbit immunogloblin G (IgG) and the colorogenic substrates BCIP/NBT. The cell lysates were also incubated with biotinylated paratropomyosin and then the binding was detected by AP-labeled streptavidin and BCIP/NBT.

Results and Discussion

The 43-kDa fragment from b-connectin/titin 2 was composed of five domains from I51 to A1 based on the human cardiac connectin/titin domain structure (Labeit and Kolmerer, 1995). As a result of expression of recombinant chicken (CK) single domains, rCK-I51, -I52, -I53 and -I54 were included in the supernatants after centrifugation of the cell lysates, but the rCK-A1 domain was precipitated. Recombinant domains in the supernatants were separated and purified by applying to column chromatography. Figure 1A shows the band patterns of the domains separated by SDS-PAGE. For overlay assay, purified domains and precipitates including the rCK-A1 domain were electrotransferred to a PVDF membrane. When the domains were overlaid with paratropomyosin and then detected with anti-paratropomyosin antiserum (Fig. 1B), any specific bands did not colored in comparison to the result without paratropomyosin (Fig. 1C). On the other hand, each purified recombinant domain was immobilized on ELISA plates and incubated with paratropomyosin to detect the binding. No significant change in the absorbance depending on paratropomyosin adding was recognized at any domains (data not shown), implying that paratropomyosin was not bound to the domains. Thus, paratropomyosin was not bound to the recombinant single connectin/titin domain, whether an assay was carried out under the denatured condition, or not.

Because all recombinant single domain were lacked the sequence of amino acid residues (we call "inter-domain sequence") connecting an adjacent domain, paratropomyosin seems not to be bound to the

domains.

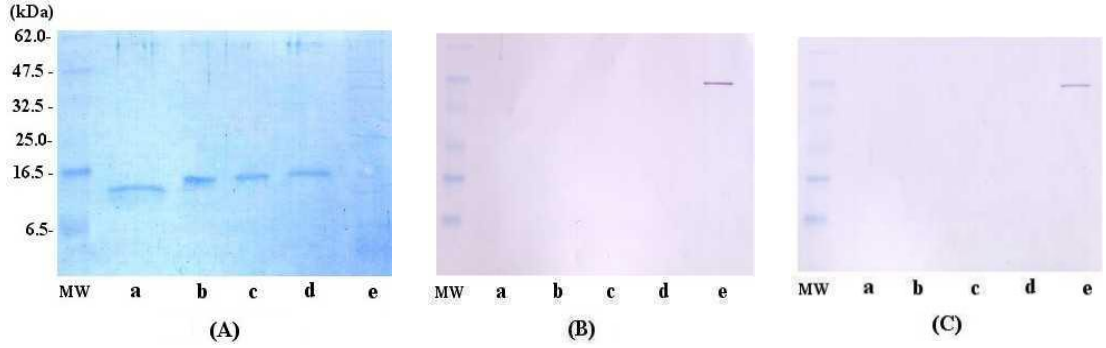

Fig. 1. Detection of binding of paratropomyosin to recombinant single connectin/titin domain by overlay assay. (A), Naphthol blue black protein stain; (B), paratropomyosin overlay; (C), control. Lanes a, b, c and d are rCK-I51, -I52, -I53 and -I54 domain, respectively and lane e is precipitates including rCK-A1 domain.

Otherwise the binding is possible to need at least two adjacent domains. So, we expressed in *E. coli* recombinant two successive connectin/titin domains, rCK-I51-I52, -I52-I53, -I53-I54 and -I54-A1, where the inter-domain sequences were included. Figure 2A shows the expression pattern of the recombinant domains after centrifugation of the cell lysates. When the domains were overlaid with paratropomyosin, the band of the rCK-I54-A1 domain was specifically colored (Fig. 2B, lane d) in comparison to the result without paratropomyosin (Fig. 2C). Also, the band of the rCK-I54-A1 domain was specifically colored when biotinylated paratropomyosin was used for the assay in place of unmodified paratropomyosin and detected by streptavidin conjugated with alkaline phosphatase (data not shown).

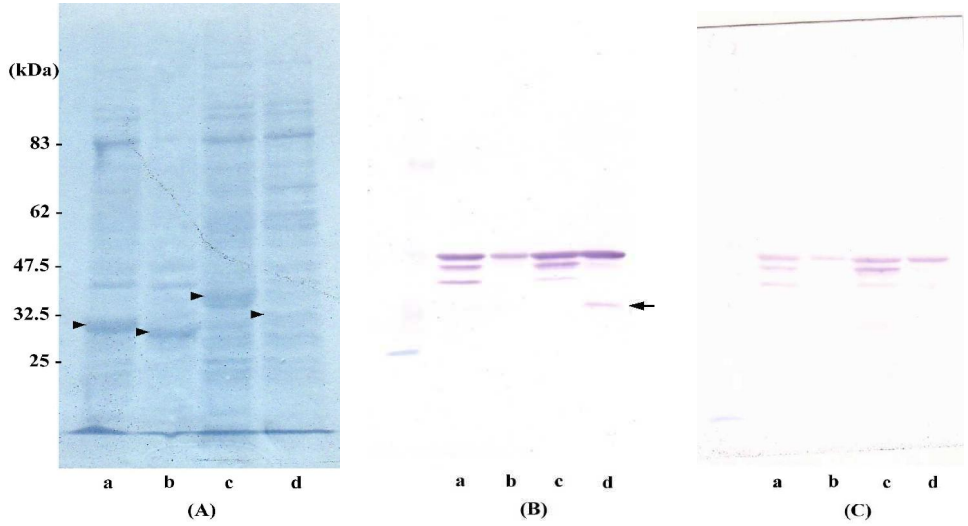

Fig. 2. Detection of binding of paratropomyosin to recombinant two successive connectin/titin domains by overlay assay. (A), Naphthol blue black protein stain; (B), paratropomyosin overlay; (C), control. Lanes a, c and d are rCK-I51-I52, -I53-I54 and -I54-A1 domain, respectively. Lane b is precipitates including rCK-I52-I53 domain. Arrow heads represent the bands of the recombinant domains. An arrow indicates the rCK-I54-A1 domain band that was specifically colored by paratropomyosin binding.

Conclusion

The results in this study suggest that paratropomyosin is bound to a site of the I54-A1 domain of the 43-kDa fragment, and that either the inter-domain sequence or both the I54 and A1 domain is necessary for the binding.

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